Kidney International, Vol. 41 (1992), pp. 1090–1105

NEPHROLOGY FORUM

Molecular pathogenesis of membranous nephropathy

Principal discussant: DONTSCHO KERJASCHKI

University of Vienna, Vienna, Austria

Case presentation

A 64-year-old university executive was admitted in 1983 to the University of Vienna Department of Surgery for the removal of hemorrhoids. He presented with slight generalized edema, and subsequently unselective proteinuria (1.4 g/day) was discovered. He was transferred to the Nephrology Section and a renal biopsy was sent to the Department of Pathology of the University of Vienna, Allgemeines Krankenhaus.

Light microscopy with serial paraffin sections revealed an even, modest thickening of all peripheral capillary loops of the entire glomerular basement membrane. The number of mesangial cells and the average area of the mesangium were normal; no inflammatory cells were detected in the glomerular tufts. Bowman's capsule and the vascular system were inconspicuous; the interstitial space was slightly edematous. Proximal tubules contained a few resorption droplets, which contained albumin and immunoglobulins on immunocytochemical examination. The tubular system appeared normal otherwise. Silver impregnation of the paraffin sections showed a serrated pattern of the basement membranes of the peripheral capillary loops that was caused by spikes extending from the subepithelial glomerular basement membrane. Immunocytochemical analysis disclosed abundant IgG, C3c, and C5b-9 in a punctate pattern in the basement membrane. Immune deposits were evenly distributed within the peripheral capillary walls and were of similar sizes; they formed a regular, discrete pattern. Weak staining for C1q was detected, but IgM, IgA, and fibrinogen were absent. There was no staining in the mesangium for any of these molecules.

Presentation of this Forum is made possible by an educational grant from Merck Sharp & Dohme International. This Forum was presented in Talloires, France, in May 1991.

Electron microscopic examination showed immune deposits in the lamina rara externa; these deposits were separated by small spikes of matrix. The deposits, which consisted of finely granular, moderately electron-dense material, were covered by podocytes that had lost their discrete foot processes and had the appearance of flat epithelial cells.

Because investigations showed no evidence of infectious or malignant disease, the lesion was classified as primary (idiopathic) membranous nephropathy, stage II (according to the Ehrenreich and Churg classification [1]). No treatment was given to the patient for the following 5 years, during which time the unselective proteinuria increased slightly, to 2.8 g/24 hrs. After 5 years, the plasma creatinine increased from 0.14 to 0.19 mmol/liter (2.1 mg/dl), and mild hypertension developed (180/95 mm Hg). At this point, triple therapy according to the schema of Ponticelli was introduced [2]; the treatment was associated with stabilization of the creatinine levels for another 1.5 years. Thereafter creatinine levels rose unremittingly, and one year ago the patient reached end-stage renal failure. He has received hemodialysis treatment since then.

Discussion

DR. DONTSCHO KERJASCHKI (*Professor of Pathology, University of Vienna, Allgemeines Krankenhaus, Vienna, Austria*): Among the numerous patterns of immune deposit formation in human glomerular autoimmune diseases, that of membranous nephropathy is particularly intriguing because it affects all glomeruli evenly, and the pathomorphologic and immunohistologic features are virtually identical among different patients. Also, the clinical course is similar in the majority of patients [1, 3–5]. The morphologic changes progress from small subepithelial immune deposits in the lamina rara externa of the glomerular basement membrane (GBM) to large, GBM-matrix-encapsulated defects in the GBM that might or might not contain immunoglobulins and complement components, depending on whether the disease is in an active stage. Approximately 50% of the patients with membranous nephropathy follow the course exemplified by today's case presentation, that is, progression to renal insufficiency over an average of 10 years, regardless of therapy [6, 7]. In many cases, immunosuppressive therapy does nothing more than retard the development of relapses of the nephrotic syndrome; it does not appear to interfere with the disease's pathogenesis, that is, the formation of immune deposits, which eventually leads to glomerular damage. It is hoped, therefore, that determining the molecular mechanisms involved in formation of the immune deposits and in the subsequent glomerular damage will enable us to develop specific "hand-tailored" diagnostic and therapeutic strategies that will improve the outcome of patients with this disease.

The availability of experimental animal models of membranous nephropathy has enabled researchers to extend our knowledge of this disease far beyond that possible from the study of

the limited renal tissue obtainable from patients. Heymann nephritis (HN) closely mimics the morphologic features and clinical course of the human disease [8]. Heymann nephritis originally was induced in rats by active immunization with homogenates of renal cortex in complete Freunds adjuvant. As the rats develop antibodies against renal antigens, immune deposits appear in the capillary loops that are indistinguishable from immune deposits in human membranous nephropathy. After 6 to 8 weeks, proteinuria develops and persists throughout the life of the animals. Because the formation of the immune deposits in this setting relies on active immunization and active production of rat IgG against an exogenous antigen, this variant of the experimental disease was named "active" Heymann nephritis.

A similar morphologic picture and the development of proteinuria within a few days can be achieved by passive transfer of autologous or heterologous anti-rat renal cortex antibodies into healthy rats [9, 10]. This variant of the disease was called "passive" Heymann nephritis because the nephritogenic antibodies are transferred passively into a normal host. Passive Heymann nephritis is a favorable system for detailed study both of one form of immune reactant deposition and of the mechanisms responsible for glomerular damage resulting in proteinuria. The features of the disease develop rapidly; the kidney as the target organ is able to concentrate even a few micrograms of specific IgG to form immune deposits within a short time of injection. The findings obtained in this model are relevant both for the active form of Heymann nephritis and possibly, at least in certain cases, for human membranous nephropathy.

In a 1989 Nephrology Forum, Verroust summarized several kinetic aspects of the formation of immune deposits in Heymann nephritis [11]. I will focus here on recent concepts of molecular mechanisms of the formation of immune deposits in Heymann nephritis.

Formation of immune deposits

Introduction and background. In membranous nephropathy, as in other examples of glomerular autoimmune disease, the deposition of immunoglobulins in the capillary loops is the principal source of damage to the filtration barrier. It soon became apparent that both in Heymann nephritis and in other glomerular diseases, the immunoglobulins are present in the form of immune complexes; an intense search for the responsible antigen or antigens therefore has been conducted. In the case of Heymann nephritis (as for several other immune complex diseases) [12, 13], antibody-antigen complexes originally were thought to form in the circulation and then to deposit in a granular pattern in the peripheral capillary loops [14, 15]. However, later evidence demonstrated that the antigen(s) involved in this disease are present within the glomerulus itself and that immune complexes form "in situ" following fixation of circulating specific antibody [16, 17]. An understanding of the mechanisms of immune deposit formation in Heymann nephritis required identification of the glomerular antigen(s) involved and a determination of their precise localization in normal and disease states.

Several attempts have been made to identify the pathogenic antigen(s) involved in Heymann nephritis. One early strategy was based on subfractionating a crude extract of rat renal cortex; the pathogenic activity was recovered in a lipoprotein fraction named Fx1A [15]. From this extract a subfraction was derived named RTEα5, in which the pathogenic potency contained in the extract of the whole-kidney cortex was concentrated [15]. Also, RTEα5 was shown to contain lipids and proteins (or fragments of proteins) but the precise composition could not be determined because accurate analytic techniques (for example, SDS-slabgel electrophoresis) were not available at the time. Both Fx1A and RTEα5 are still widely used for induction of active Heymann nephritis, and antibodies raised against these fractions have been useful in the study of passive Heymann nephritis.

These and several other attempts at purifying the nephritogenic molecule(s) were hampered by the heterogenous nature of the starting material, Fx1A, and by the variability in its composition from one preparation to another and from one laboratory to another. We now know that the brush-border region of proximal tubules contains most if not all of the pathogenic antigenicity, as shown by indirect immunofluorescence using either circulating antibodies of Fx1A-immunized rats or glomerular eluates [18].

The identification of gp330 as the pathogenic antigen in Heymann nephritis. The localization of the pathogenic antigen(s) in the proximal tubule brush border led to attempts at purifying the corresponding molecule(s) from isolated microvillar fractions that could be prepared in high purity by fractionation techniques originally used for the study of various ion and glucose transporters. One approach used microvillar fractions as a source for the production of monoclonal antibodies in the hope that one or more of these antibodies would induce passive Heymann nephritis. This goal, however, was not fully accomplished [19, 20]. In another approach, the glycoproteins of solubilized microvilli were subfractionated on lectin columns, and individual glycoprotein batches were used for the induction of active Heymann nephritis. Lentil lectin-binding glycoproteins (indicative of mannose- and fucose-containing sugar side chains) were active, but those obtained with other lectins were much less nephritogenic [21].

Concurrently, we started a series of experiments that were based on the hypothesis that the IgG in glomerular immune deposits could be used as a specific probe for identifying the corresponding antigen(s) in lysates of microvillar proteins [22]. Microvillar fractions from proximal tubules were prepared, radio-iodinated, and lysed in detergent buffer. Then IgG within glomeruli of rats with Fx1A-induced active Heymann nephritis was eluted with citrate buffer. When incubated with the lysates of microvilli, a single glycoprotein with an apparent molecular size of 330 kD was selectively immunoprecipitated; we named it gp330 (Fig. 1). Our assessment of the molecular weight of gp330 originally was based on a comparison with the mobility of molecular weight standards; our estimate has been confirmed subsequently both by cleavage of gp330 with cyanogen bromide and addition of the molecular weights of the individual fragments, and, more recently, by amino acid analysis (unpublished observations).

Subsequent studies demonstrated that gp330 is a major component of Fx1A and of RTEα5 [23]. Whether the gp330 molecule was capable of inducing Heymann nephritis by itself remained to be shown.

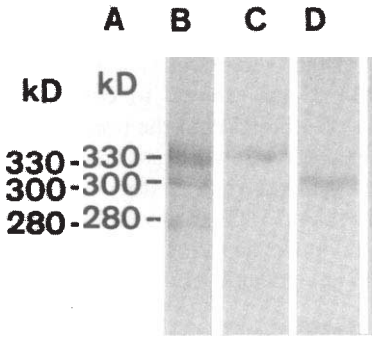

Fig. 1. *Gallery of the currently known gp330-related membrane proteins in rat proximal tubule brush border.* Immunoprecipitates of radio-iodinated rat microvillar proteins with: **A** Anti-gp330 antibodies raised in rabbit against purified rat gp330. Note that three bands are immunoprecipitated with apparent molecular weights of 330, 300, and 280 kD; **B** Monoclonal anti-gp330 IgG, which selectively recognizes the gp330 band. An identical pattern of immunoprecipitation was obtained when the glomerular IgG was eluted from rats with active or passive Heymann nephritis; **C** A monoclonal anti-maltase (gp300) antibody (a gift from Dr. B. Sacktor). In contrast to the anti-gp330 or gp280 antibodies, this IgG was able to specifically deplete a lysate of microvilli of maltase activity; **D** A rabbit anti-280 kD antibody (obtained from Dr. Ch. Leung). This antibody, which selectively recognizes the 280 kD band, is a potent teratogenic agent in rats in the first half of the pregnancy.

The induction of immune deposits by anti-gp330 antibodies. Purified gp330, obtained either by column chromatography or by electroelution from SDS-gels or by immunoaffinity purification with monoclonal anti-gp330 antibodies, was used to immunize susceptible rat strains. Within 4 to 6 weeks, glomerular subepithelial immune deposits were found; these contained endogenous rat IgG and gp330 antigen [22]. The glomerular IgG was acid-eluted and was monospecific for gp330, as determined by immunoprecipitation. These data thus indicate that subepithelial immune deposits similar to those caused by Fx1A preparations can be induced by purified gp330.

When monospecific anti-gp330 antibodies were prepared by affinity purification on immobilized purified gp330 from rabbit or sheep anti-rat Fx1A IgG, and were injected intravenously into rats, subepithelial immune deposits developed within minutes in clathrin-coated pits on the "soles" of the podocytes [24] (Figs. 2 and 3). A major difference remains, however, between the functional abnormalities associated with these immune deposits and those associated with deposits induced by anti-Fx1A IgG; proteinuria was much less prominent or even absent when monospecific anti-gp330 IgG was used. This observation indicates that at least one factor in addition to gp330 immune complexes is necessary for the development of "full-blown" Heymann nephritis with immune deposits and proteinuria.

Antigens other than gp330. When IgG was eluted from glomeruli with Fx1A-induced active or passive Heymann nephritis, some investigators discovered that, in addition to gp330, other molecules also were tagged by the antibodies, as detected either by immunoblotting or immunoprecipitation of microvillar proteins [25–30]. This finding was interpreted as meaning that molecules other than gp330 could contribute to the formation of immune deposits and/or proteinuria. Subsequently one of these molecules was identified as the enzyme dipeptidyl-peptidase IV (DPP IV); intravenous injection of monospecific anti-DPP IV antibodies gave rise to transient glomerular immune deposits and—at least in one laboratory—also caused proteinuria [11, 31–35]. To clarify the role of this enzyme, we mixed monospecific anti-gp330 antibodies with anti-DPP IV antibodies (obtained from Dr. P. Verroust) raised in a different species; we then injected the mixture into rats. When followed separately by immunoelectron microscopy, the two IgGs did not co-localize in the immune deposits. Similar results were obtained when anti-gp330 and anti-laminin antibodies were mixed. These data indicate that neither anti-DPP IV nor anti-laminin IgG participates in the formation of immune deposits in passive Heymann nephritis. It is possible, however, that DPP IV immune complexes assist indirectly in the development of gp330-dependent immune deposits [11].

To examine the putative multispecificity of the immunoglobulin within immune deposits in Heymann nephritis in a systematic way, we recently obtained sheep Fx1A IgG (provided by Dr. W. G. Couser, and purified by protein G affinity chromatography) and depleted it completely of its anti-gp330 activity by passing it repeatedly over a cyanogen bromide-Sepharose column to which purified gp330 was immobilized. When the monospecific anti-gp330 IgG from the column was injected into rats, typical subepithelial immune deposits developed, whereas the anti-gp330 IgG-depleted anti-Fx1A serum failed to bind to the glomeruli at all time points studied (from 15 minutes to 7 days after injection; Susani M, Exner M, Kerjaschki D, unpublished observations). These experiments clearly indicate that in passive Heymann nephritis, anti-gp330 antibodies are the only fraction of IgG in anti-Fx1A serum capable of forming stable immune deposits, whereas all the other IgG specificities contained in this serum are not able to do so. Corresponding experiments also were performed previously in active Heymann nephritis [22]; isolated rat microvilli were depleted of gp330 and used for immunization of rats. Under these conditions, no immune deposits were formed, but purified gp330 gave a positive result.

Several laboratories have confirmed these findings [19, 25, 28, 36–40]. Others, however, have contested the data and have described other candidate antigens, which apparently are different from gp330, or of which gp330 is only a component, such as gp600 [41]. For example, anti-gp600 IgG subsequently was found to be multispecific with a major specificity for gp330, but also for several other high-molecular-weight proteins as resolved by gel filtration of Fx1A. In active Heymann nephritis, some investigators have found circulating immune complexes [42–44], but the direct nephritogenicity of these complexes has not been convincingly proved [45]. In one particular case, a molecule was isolated from these immune complexes that induced immune deposits when used for active immunization of rats [46]. It is not yet clear whether such antigens in circulating immune complexes are related to gp300.

Taken together, the available data indicate that gp330 is the sole antigen present in Fx1A preparations (and presumably also in RTEα5) capable of forming stable and persistent immune deposits in the lamina rara externa in passive and presumably also in active Heymann nephritis. These findings warrant a closer inspection of the properties of the gp330 molecule with particular reference to how its molecular structure could relate to the formation of immune deposits.

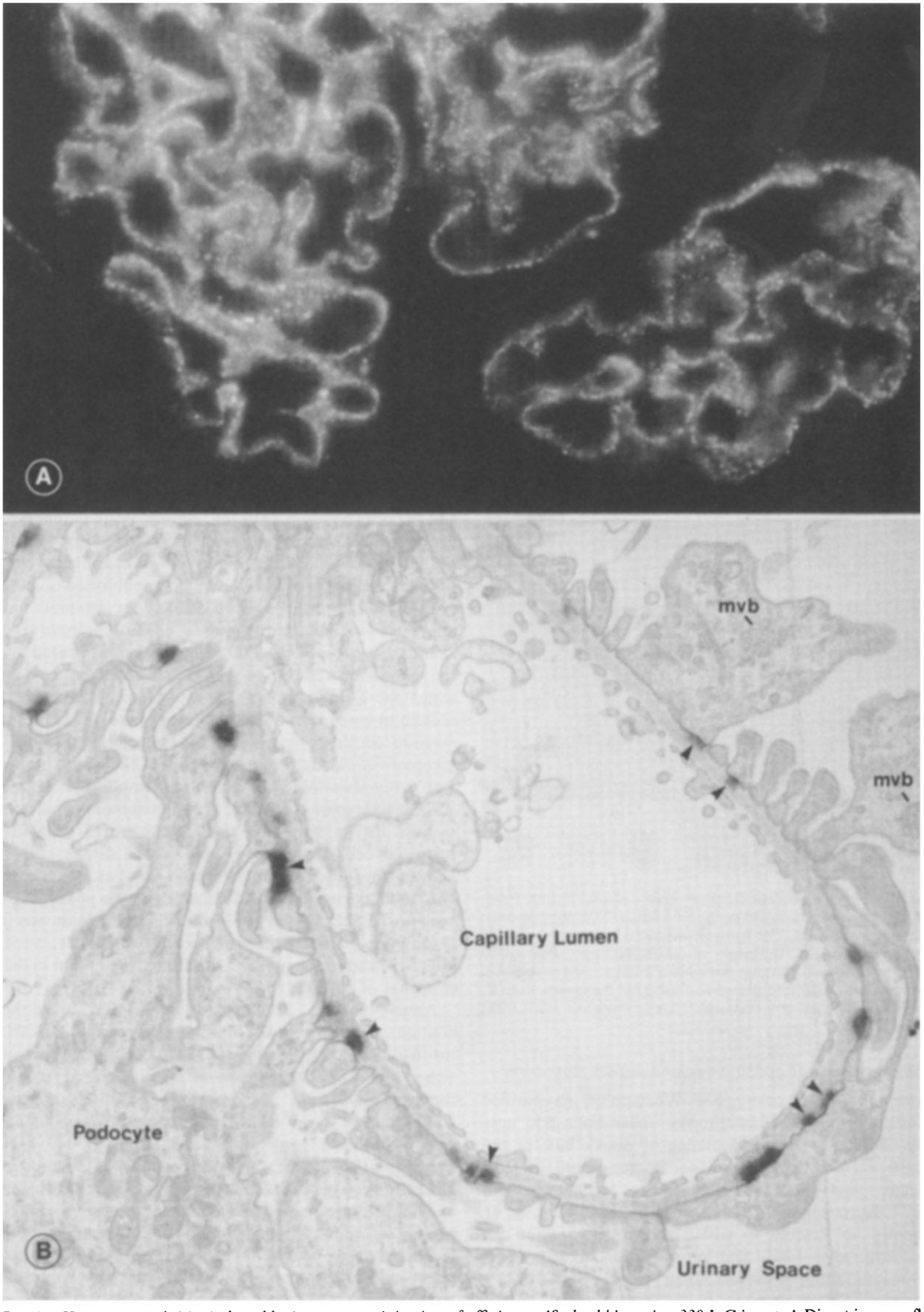

Fig. 2. *Passive Heymann nephritis, induced by intravenous injection of affinity-purified rabbit anti-gp330 IgG in rat.* **A** Direct immunofluoresence showing granular deposits in the peripheral capillary loops; the deposits contain the injected rabbit anti-gp330 antibodies. **B** Immunoelectron microscopy using immunoperoxidase for the detection of injected rabbit IgG 6 days after intravenous injection of affinity purified rabbit anti-gp330 IgG into a rat. The arrows show immune deposits that contain reaction product indicative of rabbit IgG in typical subepithelial localization in the lamina rara externa. In many instances the immune deposits can be seen attached to the basal cell membrane of the podocytes rather than between the slit diaphragms. mvb: multivesicular body. (**A** × 1500, **B** × 25,000.)

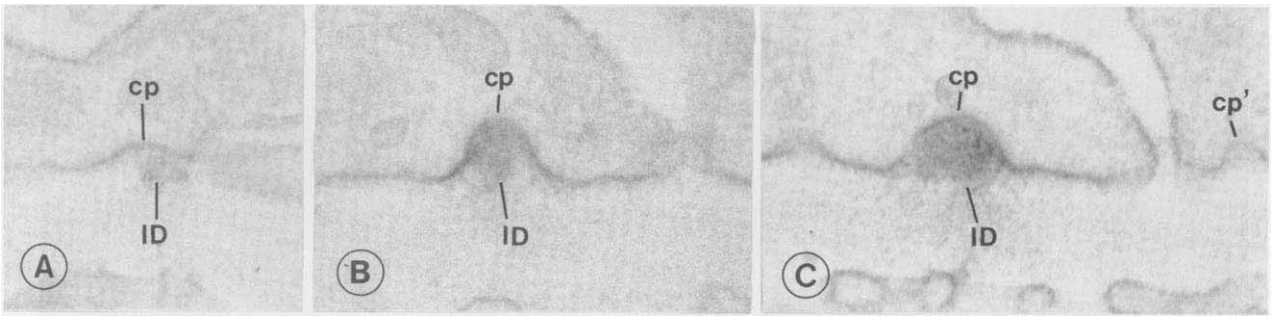

Fig. 3. *Early stages of passive Heymann nephritis induced by intravenous injection of monospecific rabbit anti-gp330 IgG.* After 15 minutes (**A**), 1 hour (**B**), and 1 day (**C**), the injected rabbit IgG is found selectively in coated pits (cp) on the "soles" of the foot processes, and forms small immune deposits (ID). This is precisely the same location at which gp330 was found previously; the initial immune complexes and early events in the formation of immune deposits thus likely are tightly associated with clathrin-coated membrane areas of the podocyte membrane. Note that not all coated pits in this region contain anti-gp330 immune complexes (cp' in C). (Magnification × 75,000.)

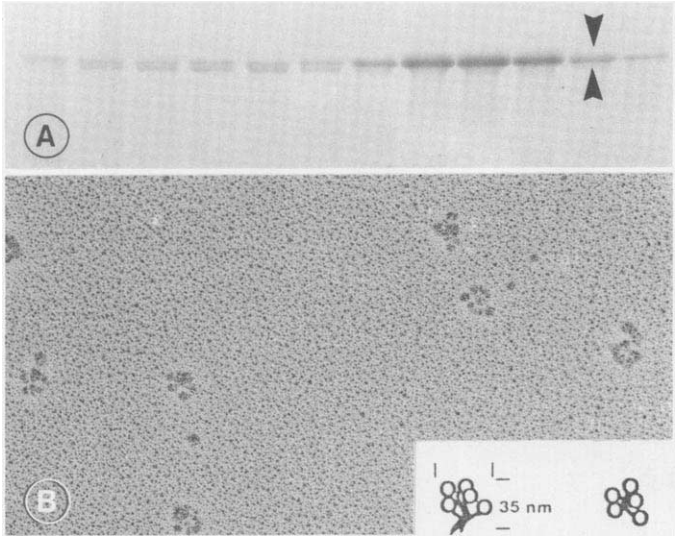

Fig. 4. *The shape of gp330 visualized by rotary shadowing.* **A** Fractions of gel filtration column resolving gp330 and gp300 in the presence of 1% sodium deoxycholate. The fraction indicated by arrowheads was used for rotary shadowing. **B** Images of gp330 indicating that gp330 forms aggregates in which globular domains are associated with stalk-like regions (outlined in the interpretative drawing in the insert, Kain R, Kerjaschki D, unpublished observations). (**B** magnification × 201,600.)

Molecular analysis of gp330 immune complex formation

Molecular characteristics of gp330. When gp330 was purified by high-resolution gel chromatography under bona fide non-denaturing conditions (in the presence of deoxycholate) and then visualized by rotary shadowing, it was found to form supramolecular aggregates with stalk-like regions and globular tops (Kain R, Kerjaschki D, unpublished observations) (Fig. 4). It is currently not known to which topographic region the pathogenic antibodies bind, and whether this binding could cause distortions of the molecular shape.

On the extracellular domain, there appear to be several N-linked sugar chains, although no further detailed information is available at the moment. The supposition that glycosylation occurs is based on the binding of lectins specific for mannose-containing sugar chains (as shown by Con-A binding) and fucosylation (as shown by binding of lentil lectin) [22]. It should be noted that gp330 appears to be identical to the molecule named "brushin," which was discovered in mouse embryos and teratocarcinoma cells by affinity purification on a Ulex Europaeus-lectin column, which binds to fucose [47]. By amino acid sequencing, several putative N-linked glycosylation sites were discovered recently (Binder S, unpublished observations).

The biologic function of gp330. Several attempts have been made to elucidate the function of gp330 in normal tissue. For example, the localization of gp330 in clathrin-coated pits of the cell membranes suggests that gp330 could be a receptor for which the ligands are unknown, or a constitutive membrane protein in coated pits [24, 48]. Recent binding studies of serum proteins on denatured gp330 have suggested that gp330 is a receptor for plasminogen, but proof of this claim by the use of native non-denatured molecules as ligands was not provided [49]. Other experiments have shown that monoclonal antibodies specific for gp330 inhibit the attachment of isolated tubular epithelial cells to matrix-protein-coated dishes [50], and the authors concluded that gp330 could serve as a matrix receptor. However, these studies did not exclude the possibility that the inhibitory effect of anti-gp330 on cell attachment was indirect. In addition, defined matrix receptor molecules, such as β_1-integrins, show a completely different distribution in the kidney than that of gp330 [51].

Although amino acid sequence data have revealed that gp330 contains animal lectin-like domains [52] as well as LDL-receptor-like cystine-rich domains [53], there is no direct proof for either function. Comparison of all confirmed sequence data further excludes the possibility that gp330 is identical to the LDL receptor or the LDL-related molecule (Binder S, Kerjaschki D, unpublished data).

Apparently, gp330 is only one member in a larger family of structurally related glycoproteins that currently includes the enzyme maltase, which has a molecular weight of 300 kD (and was therefore named gp300 in our previous studies) (Fig. 1). It is questionable whether the maltase-splitting activity of this gp300 molecule is of any biologic relevance in proximal tubules, because bona fide maltase is never available at this site [54]. It is currently unexplained why molecules of maltase in the proximal tubular brush border are associated exclusively with the membranes of the microvilli, whereas gp330 is present exclusively in the inter-microvillar microdomain of the cell membrane, which is clathrin-coated (Fig. 5); also unexplained is

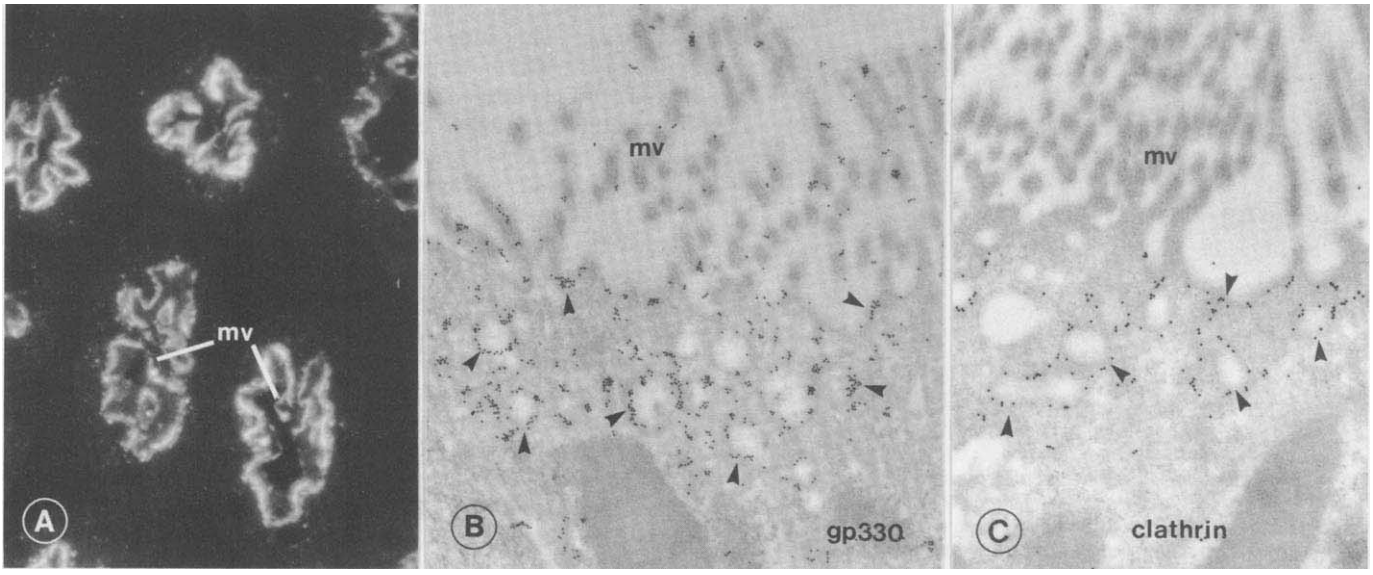

Fig. 5. *Localization of gp330 and clathrin in proximal tubule brush borders using monoclonal anti-gp330 IgG* (Figs. **A** and **B**) *and affinity-purified rabbit anti-clathrin IgG* (Fig. **C** obtained from Dr. D. Louvard). **A** Gp330 is localized at the base of the microvilli of proximal tubules and some granular compartment in the apical region of the proximal tubular cells rather than to the brush border microvilli themselves by indirect immunofluorescence with monoclonal anti-gp330 IgG. **B** By immunoelectron microscopy (Lowicryl K4M), gp330 is localized primarily on the inter-microvillar membrane of proximal tubular brush borders, and on the luminal side of apical vesicles in the proximal tubular epithelial cells. Only a few gold grains are present on the membranes of the microvilli. **C** Localization of clathrin, indicating that both the inter-microvillar microdomain as well as the apical vesicles are endowed with large amounts of clathrin. (**A** × 576; **B** and **C** × 23,040.)

how this polarity is maintained [54, 55]. Another member of the gp330 familiy is a brush-border glycoprotein with an apparent molecular weight of 280 kD in SDS gels. This molecule is of interest because specific antibodies against it cause fetal malformations in rats [56, 57]. Thus despite much effort, the biologic function of gp330 remains elusive.

"Pathogenic epitopes" on gp330. Several findings indicate that gp330 expresses "pathogenic epitopes" that form immune complexes with site-specific antibodies in vivo, thereby leading to the development of an immune deposit. By contrast, other antibody-binding sites on (denatured) gp330 are not competent.

One such piece of evidence is derived from the injection into rats of various monoclonal antibodies, either alone or in combination, that either do not bind to the glomerulus at all or form small immune deposits that disappear within a few hours [58]. However, some of these monoclonal antibodies strongly bind to immune deposits when they have been established previously by injection of, for instance, anti-gp330 antibodies or anti-Fx1A IgG. This finding suggests that more epitopes of gp330 become exposed as the immune deposits develop (unpublished observations). Second, when the glomerular IgG, eluted from animals that had been injected with affinity-purified rabbit anti-gp330 IgG three days before sacrificing, was subjected to isoelectric focusing, only a few isoelectric variants of the IgG were concentrated in the immune deposits, the original affinity-purified IgG being composed of a wide spectrum of isoelectric variants [59]. This finding indicates that a small number of presumably epitope-specific variants are selected and concentrated into early immune deposits.

A molecular biologic approach to the identification of "pathogenic epitopes" on gp330. The existence of "pathogenic epitopes" that are able to initiate immune deposits in vivo has been established by experiments in which fragments of the gp330 gene have been identified in cDNA libraries, and the corresponding fusion proteins have been expressed and probed for the presence of "pathogenic epitopes" [52]. We have identified a cDNA-clone, designated as C14, by screening a rat cDNA library with eluted glomerular IgG from animals with passive Heymann nephritis for 3 days; these animals were similar to those used for the initial identification of gp330 (Fig. 6). The amino acid sequence of C14 was determined from the nucleotide sequence (Fig. 7) and was found to contain four repeated tryptophan-containing motifs, previously observed in the ligand-binding domain of various animal lectins, such as lung surfactant and chicken hepatic lectin. The authenticity of C14 as a gp330-derived clone was established by either immunizing rats with purified C14-β-galactosidase fusion protein, or by injecting rats with monospecific anti-C14 IgG. In both instances, the observation of subepithelial immune deposits (Fig. 8) indicated the presence of at least one "pathogenic epitope" on C14 [52]. To determine the minimal amino acid sequence of this "pathogenic epitope," we are currently doing work to produce deletions in the C14 DNA, thus yielding smaller fragments of this DNA.

Currently, approximately one-third of the entire amino acid sequence of gp330 has been elucidated, including one segment at its N-terminus, which contains multiple cystine-rich motifs of the LDL receptor [53], in addition to several other fragments of gp330, which do not show any homology to other proteins. We expect that several other "pathogenic epitopes" will surface as more of the amino acid sequence of gp330 becomes known.

Although the number of "pathogenic epitopes" defined by this strategy is relatively small, it currently is not possible to

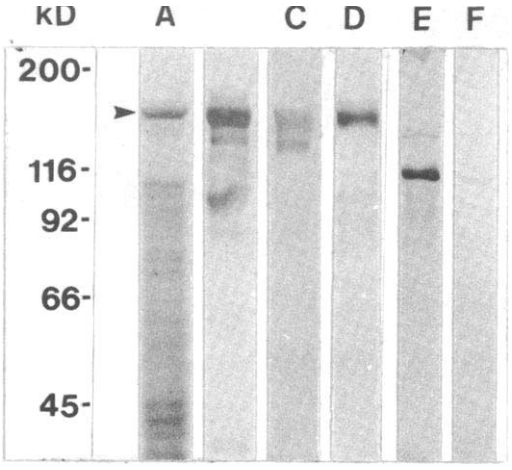

Fig. 6. *Identification of a "pathogenic epitope" of gp330 by immunoblotting on nitrocellulose transfers.* This gp330 epitope is expressed as a recombinant protein, constructed by fusion of a fragment of the gp330 gene (derived from a lambda-GT11 cDNA-library of rat kidney) with *E. coli* beta-galactosidase. *Lane A*: Lysate of *E. coli* containing a gp330 gene insert and forced to express it as a fusion protein with beta-galactosidase. In this transfer, which is stained for proteins, a prominent fusion protein (*arrowhead*) is visible. *Lane B*: Immunoblot on the proteins depicted in the preceding lane with monoclonal anti-beta-galactosidase IgG. The fusion protein and some of its degradation products are intensively stained. *Lane C*: Immunoblotting with affinity-purified anti-gp330 IgG. Note that the fusion protein and the degradation product are stained. *Lane D*: Immunoblot with IgG that was eluted from glomeruli of rats with passive Heymann nephritis. Note that the fusion protein is selectively stained. This indicates that in this fusion protein a fragment of gp330 is expressed that serves as an antigenic target in Heymann nephritis. *Lane E*: In a control experiment, *E. coli* lysate without DNA inserts was immunoblotted with a monoclonal anti-β-galactosidase IgG, which selectively stains the native enzyme with an apparent molecular weight of 160 kD. *Lane F*: When the same lysate is immunoblotted with anti-gp330 IgG, no signal is obtained.

predict whether this experimental approach will reveal a comprehensive catalogue of all "pathogenic epitopes," because epitopes dependent on the correct folding of gp330 might not be present on the β-galactosidase fusion proteins. It would be advantageous for us to know the amino acid sequence of all relevant epitopes of gp330, because this knowledge could set the stage for novel specific therapies for glomerular immune-complex diseases similar to those used in other autoimmune diseases [60, 61].

Mechanisms of the formation of immune deposits

Localization of gp330 in the normal glomerulus and other tissues. The in-situ formation of immune deposits calls for the presence of the pathogenic antigen within the glomerulus, where it meets with specific IgG from the circulation to form an initial immune complex, which subsequently grows to an immune deposit. Previous experiments using various preparations of antibodies for the localization of the Heymann nephritis antigen(s) have suggested that the antigenic targets are present either in the GBM alone [62] or in the GBM as well as along the cell membranes of endothelial and epithelial cells in an even distribution [17]. The availability of monoclonal anti-gp330 antibodies and affinity-purified polyclonal anti-gp330 IgG has clarified this issue. Found exclusively in glomerular epithelial cells, gp330 appears in the endoplasmic reticulum and the Golgi apparatus, indicating that gp330 is a biosynthetic product of these cells. On the cell surface, gp330 was exclusively associated with clathrin-coated pits, both on the urinary surface of glomerular epithelial cells, and most notably also on the "soles" of the podocytes, that is, the cell membrane that faces the glomerular basement membrane [24].

In subsequent experiments, gp330 was localized in several other tissues by immunoelectron microscopy and was exclusively present in clathrin-coated pits, which are endocytic organelles of cells specialized for the uptake of various ligands by specific receptor proteins, such as the LDL receptor [63]. For example, in renal proximal tubules, gp330 was found exclusively in the clathrin-coated inter-microvillar microdomains of the brush-border membranes [54] (Fig. 5). The association between clathrin and gp330 is so strong that it is maintained even when the brush-border membranes are isolated by biochemical fractionation techniques [55]. Also, gp330 was found in coated pits in the apical membranes of epididymal epithelial cells, in the rat yolk-sac and type-II pneumocytes, in the epithelial cells of ependyma, and in prospective epithelial cells of early mouse embryos [38, 64, 65].

Formation of immune deposits in coated pits of podocytes. When polyclonal affinity-purified anti-gp330 IgG was injected intravenously into normal rats, glomerular subepithelial immune deposits appeared within 10 minutes. These deposits were found by immunoelectron microscopy exclusively within the clathrin-coated pits of the glomerular epithelial cells in the lamina rara externa (Fig. 3). With time, the immune deposits increased in size but always remained in contact with the clathrin-coated areas of the podocytes even after several weeks, presumably because newly formed gp330 is presented there [59].

Immune deposits firmly adhere to the GBM. The association of immune deposits with coated pits is intriguing because these organelles are sites of endocytosis, yet the gp330 immune complexes were allowed to accumulate there without being taken up and metabolized by the cell. This phenomenon was explained by the finding that gp330 immune complexes are immobilized by attachment to the lamina rara externa of the GBM as early as 15 minutes after intravenous injection of anti-gp330 IgG, and the complexes could not be removed by extraction with detergents, nor by high- and low-salt buffers, such as those used for the purification of GBM (Fig. 9). By contrast, the finding that gp330 itself was not detected in the isolated GBMs indicates that this attachment is specific for gp330 immune complexes only [59]. The molecular mechanism accounting for this process, and whether this interaction is indiscriminate or is selective for individual matrix molecules, is not known. A similar mechanism conceivably could account for the formation of stable immune deposits not only in Heymann nephritis, but also in other glomerular immune complex diseases.

Shedding of gp330 immune complexes from the surface of podocytes. When cultured glomerular epithelial cells are exposed to anti-gp330 antibodies to form immune complexes in their coated pits, these complexes are primarily taken up by the cells via endocytosis. Apparently, however, the complexes also are partially shed from the cell surface and released into the medium [67–69]. Immunohistochemistry studies documented

```
    R  S  A  E  K  N  E  P  E  M  A  A  K  R  E  S  G  E  E  F  R  M  E  K  L  N  Q  L  W  E  K  A  K  R  L  H  L  S
    CGCTCGGCGGAGAAGAATGAGCCCGAGATGGCCGCCAAGCGTGAGTCCGGGGAGGAGTTCCGCATGGAGAAGCTGAACCAGCTATGGGAGAAGGCCAAGCGGCTGCATCTGTCT

    P  V  R  L  A  E  L  H  S  D  L  K  I  Q  E  R  D  E  L  N  W  K  K  L  K  V  E  G  L  D  G  D  G  E  K  E  A  K
115 CCTGTGAGGCTGGCCGAGCTGCATTCTGACCTGAAGATACAAGAGAGGGATGAACTCAACTGGAAGAAGCTGAAGGTGGAAGGCCTAGACGGGGATGGGGAGAAAGAAGCAAAA

    L  V  H  N  L  N  V  I  L  A  R  Y  G  L  D  G  R  K  D  T  Q  T  V  H  S  N  A  L  N  E  D  T  Q  D  E  L  G  D
229 CTGGTCCACAATCTCAATGTCATCCTGGCCAGGTACGGACTGGATGGGAGGAAGGACACCCAGACAGTGCACAGCAACGCCCTCAATGAAGACACCCAGGATGAGCTGGGGGAC

    P  P  L  E  K  L  W  H  K  A  K  T  S  G  I  S  V  R  L  T  S  C  A  R  V  L  H  Y  K  E  K  I  H  E  Y  N  V  L
343 CCCAGGTTGGAAAAGCTGTGGCACAAGGCAAAGACATCAGGAATCTCAGTGAGACTGACAAGCTGTGCGAGAGTTCTGCACTACAAAGAGAAGATCCACGAGTACAATGTACTG

    L  D  T  L  S  R  A  E  E  G  Y  E  N  L  L  S  P  S  D  M  T  H  I  K  S  D  T  L  A  S  K  H  S  E  L  K  D  R
457 CTAGATACACTGAGCAGAGCTGAAGAAGGTTATGAGAACCTTCTCAGCCCCTCTGACATGACCCACATCAAGAGTGACACCCTGGCCAGCAAGCACAGTGAACTGAAGGACAGA

    L  R  S  I  N  Q  G  L  D  R  L  R  K  V  S  H  Q  L  R  P  A  T  E  F  E  E  P  R  V  I  D  L  W  D  L  A  Q  S
571 CTGCGTAGTATCAACCAGGGCCTAGACCGCCTGAGGAAGGTCAGCCACCAGCTACGCCCCGCCACTGAGTTTGAAGAGCCCCGAGTGATAGATCTGTGGGACCTGGCTCAGTCT

    A  N  F  T  E  K  E  L  E  S  F  R  E  E  L  K  H  F  E  A  K  I  E  K  H  N  H  Y  Q  K  Q  L  E  I  S  H  Q  K
684 GCCAACTTCACCGAGAAGGAACTGGAGTCGTTCAGGGAGGAGCTTAAGCACTTCGAGGCCAAAATCGAAAAGCACAACCACTACCAGAAGCAGCTGGAGATTTCCCACCAGAAG

    L  K  H  V  E  S  I  G  D  P  E  H  I  S  R  N  K  E  K  Y  V  L  L  E  E  K  T  K  E  L  G  Y  K  V  K  K  H  L
799 CTGAAGCACGTGGAGAGCATCGGTGACCCCGAGCACATCAGCCGCAACAAGGAGAAGTACGTCCTGCTGGAGGAGAAGACAAAGGAGCTGGGCTACAAGGTGAAGAAGCATCTG

    Q  D  L  S  S  R  V  S  R  A  R  H  N  E  L
912 CAGGACCTGTCCAGCAGGGTCTCAAGGGCTCGGCACAATGAGCTCTGAGACCAGAAGCCACTGGCAGTAGCCTAGAGAGGCTCTTGAAGACACTGGGAACTGTCAACTTGTCAT

1027 ACTGGTGTGGCTGTCCACAGTGGCAAGGAGAAATACCGATCTGATCCTGCTGCGGCTGGCAAGGACTAATTTTCTTTCAAGCAAGTGTAGCTGTCACCGCCCTGGTTGAGGGCT

1141 TTGGGTACATCTACCAATGAGATATGACCGGTCTCCATGCTGCAGCAAAAGTTTACATTGAAATTATATAAATCTGTCACTGGAAATGTTATGTACAGAGTCCTTAAATACATG

1255 GCAGCG
```

Fig. 7. *Nucleotide sequence and derived amino acid sequence of the DNA insert of gp330, which was used for production of subepithelial immune deposits and which bound the eluted glomerular IgG of rats with passive Heymann nephritis.* This fusion protein represents the C-terminal portion of the gp330 molecule. It contains one potential glycosylation site and four repeating tryptophan-containing regions (*boxed areas*), which are very similar or identical to conserved domains found in the ligand-binding region of various animal lectins. This insert contains about 280 amino acids.

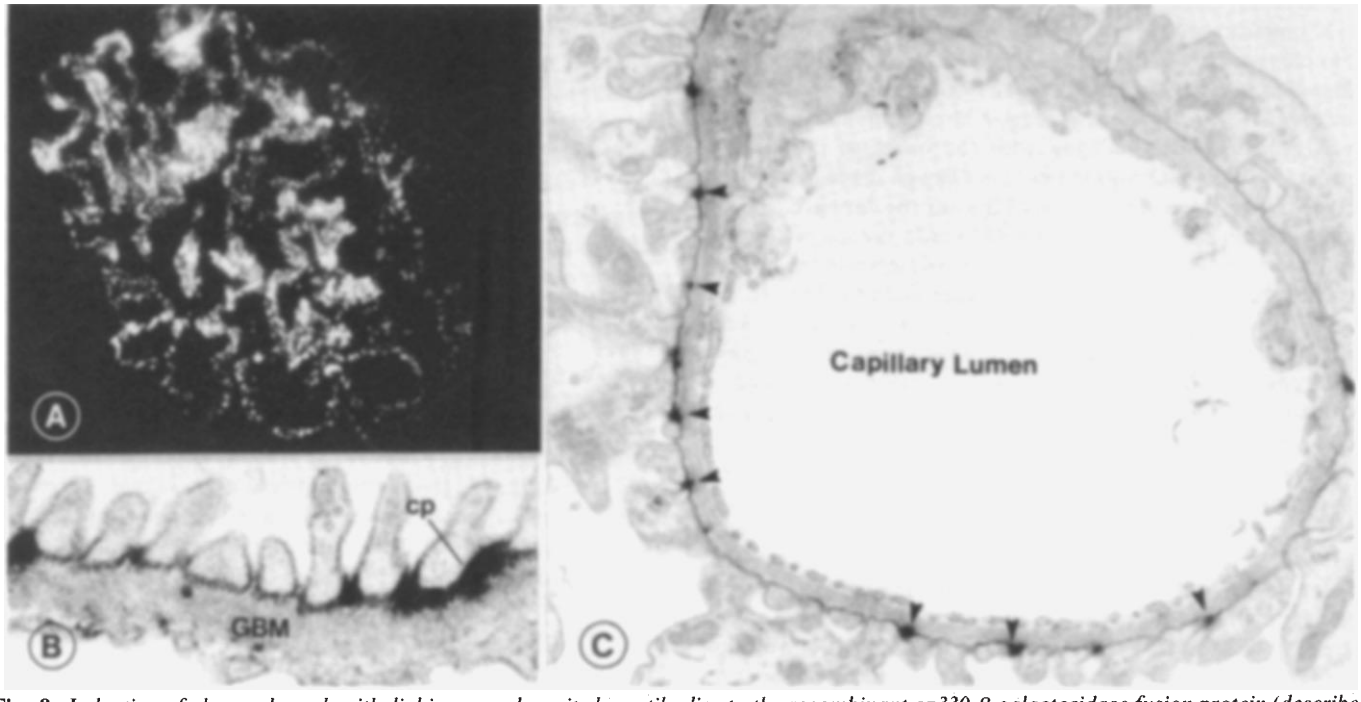

Fig. 8. *Induction of glomerular subepithelial immune deposits by antibodies to the recombinant gp330-β-galactosidase fusion protein (described in the preceding figure).* **A** When this IgG is injected intravenously into normal rats, granular immune deposits in the glomerular capillary walls are observed by direct immunofluorescence. **B, C** Six weeks after this fusion protein was injected to induce active immunization of rats, granular immune deposits developed; the deposits contain rat IgG (as visualized by direct immunoperoxidase immunoelectron microscopy). (**A** × 500, **B** × 26,000, **C** × 17,000).

patching, capping, and shedding of gp330 immune complexes; these changes resembled similar processes on B-lymphocytes after incubation with anti-immunoglobulin antibodies [67, 68]. A similar detachment process also could occur in the glomerulus after injection of anti-gp330 IgG, and this detachment could facilitate the anchoring of immune complexes in the GBM. It is remarkable that chlorpromazine, which is known to interfere with rearrangement of cell surface molecules by influencing the

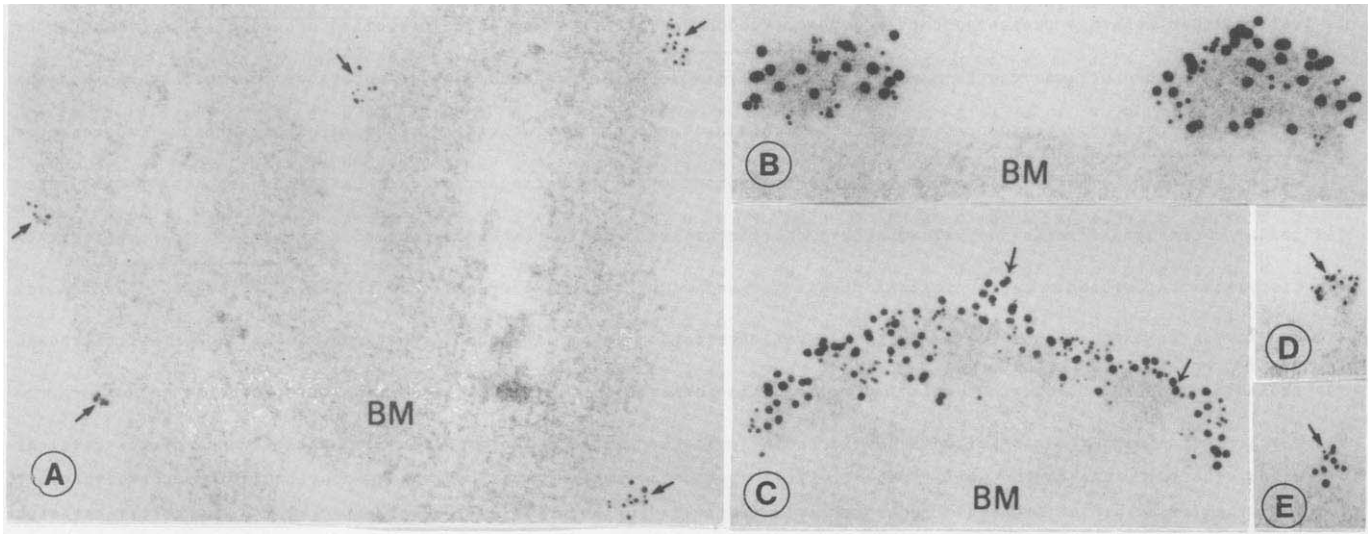

Fig. 9. *Gp330 immune complexes are rapidly immobilized in the glomerular basement membrane.* Anti-gp330 IgG was injected intravenously into rats, and the glomerular basement membranes were isolated by extraction with detergents. Both gp330 (small gold particles) and the injected IgG (large particles) were localized simultaneously by immunoelectron microscopy. At 15 minutes (**A**) and, more pronounced, at 3 days after injection (**B-E**), both components of the immune deposits stick firmly to the lamina rara externa, even when the cells have been extracted. (**A** × 83,300; **B** × 117,600; **C** and **D** × 83,300; **E** × 93,100.)

cytoskeleton, inhibits (both in vitro and in vivo) the shedding of gp330 immune complexes and the formation of immune deposits in passive Heymann nephritis respectively [69, 70].

Immune deposits in tubular basement membranes in active Heymann nephritis. A few weeks after immunization with Fx1A antigen and coinciding with the onset of proteinuria, anti-Fx1A autologous antibodies leak through damaged glomeruli and are exposed to their antigens on the brush border of proximal tubules. This leakage results in a complement-independent, transient damage to the proximal tubules, which includes the loss of microvilli and a flattening of cells [71]. We also found that gp330 is no longer expressed with restricted polarity on the luminal side of the tubular epithelial cells under these circumstances, but also appears basolaterally and in particular on the basal surface of the tubular cells, facing the tubular basement membrane (Kerjaschki D, Noble B, Andres GA, unpublished data). Immunoelectron microscopy has revealed that immune deposits on the tubular basement membrane are located within the coated pits of the modified tubular epithelial cells (Fig. 10); this finding suggests a mechanism similar to that of the formation of the glomerular immune deposits in Heymann nephritis [10]. Within a few weeks after the proximal tubules become distorted, the morphology of their epithelial cells is fully restored to normal. It therefore appears that the molecular mechanisms responsible for the loss of polarity have been reversed. These mechanisms are not understood, however.

Conclusions and outlook

The formation of immune deposits in Heymann nephritis is a complex process about which several—but not all—steps are understood. The currently known facts about this process can be summarized as follows (Fig. 11): Anti-gp330 antibodies traverse the glomerular basement membrane to form immune complexes in the coated pits of glomerular epithelial cells, where gp330 is exposed. Specific "pathogenic epitopes" of gp330 must be recognized by these antibodies to initiate the formation of immune complexes. This phase is followed by anchoring of the gp330 immune complexes to the GBM, and detachment from the surface of the podocyte cell membrane. The glomerular epithelial cells respond by increasing the rate of synthesis of gp330. Vesicular transport of these new molecules of gp330 exposes them in the membranes of coated pits adjacent to immune deposits. By repeated cycles of this process, more immune complexes can accumulate at the same spot, and eventually the immune deposits become morphologically apparent. Theoretically, several strategies could be designed to interrupt this chain of events and halt the disease, for example, the provision of synthetic antigen decoys that would compete for the binding of antibodies to "pathogenic epitopes."

It remains to be seen, however, what aspects of this chain of events are present in human membranous nephropathy. There is a gp330-related glycoprotein present in human proximal tubules, but we have shown that it is absent from normal glomeruli, and it is not detectable in immune deposits in renal biopsies from patients with membranous nephropathy [72]. Several attempts by others have found some similarities between Heymann nephritis and individual cases of membranous nephropathy [73–77], but at the molecular level none of the pathogenic antigen(s) has emerged [77]. These data raise the unsettling possibility that the euphemistic term "idiopathic" will remain with us for the majority of cases of membranous nephropathy, such as the one presented here. Further study is required to establish whether a single antigen provides the target for all cases of human membranous nephropathy or whether different antigens are involved in different circumstances. Clearly, demonstrating the relevance, or lack thereof, of the findings obtained in experimental Heymann nephritis to human disease is pressing because, after all, understanding and treating the human disease remains the ultimate goal and justification for studying animal model systems.

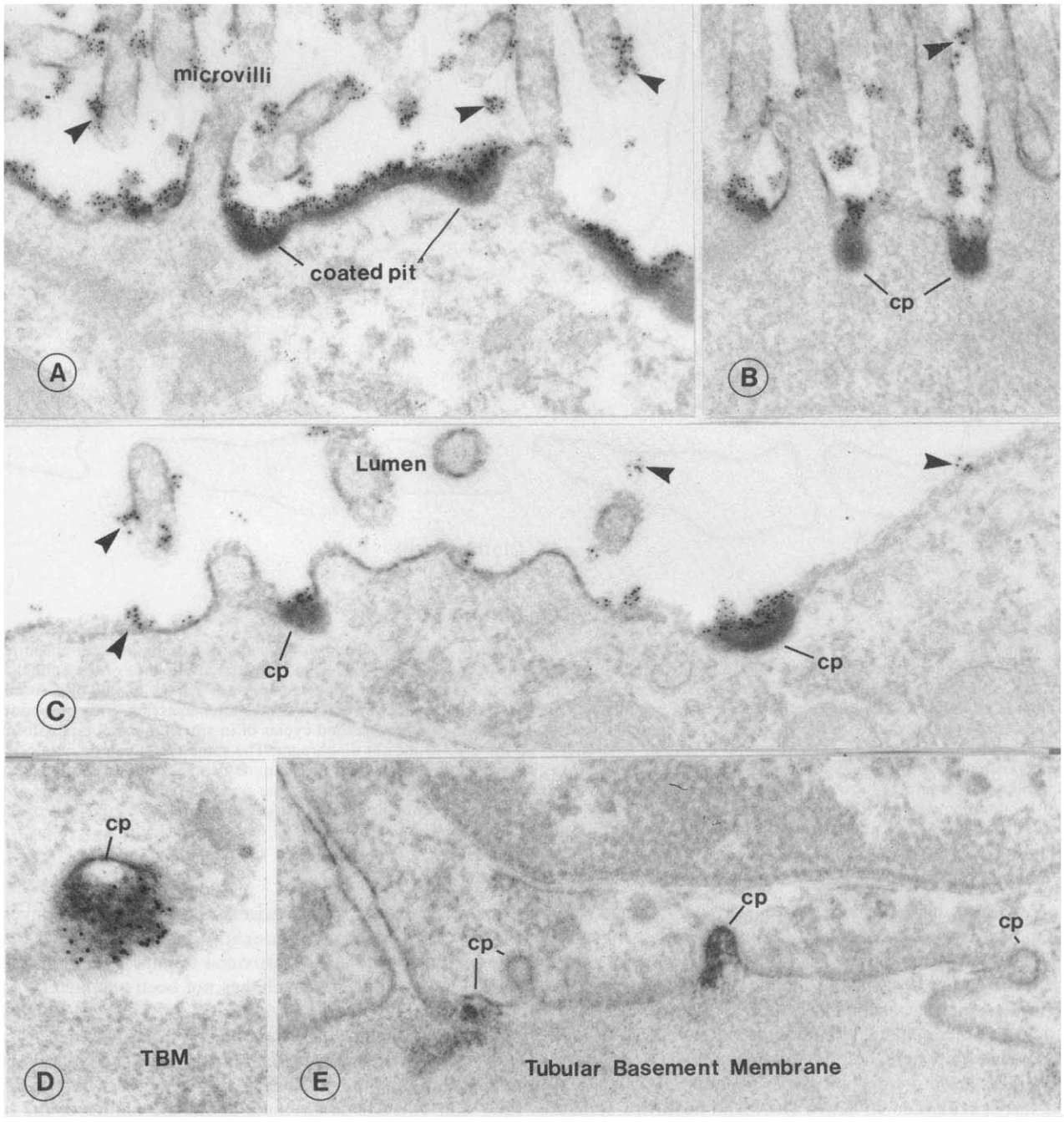

Fig. 10. *Damaged proximal tubule epithelial cells of a proteinuric rat with active Heymann nephritis* (tissue supplied by Dr. G. A. Andres). Co-localization of gp330 with a monospecific rabbit antibody (indicated by immunoperoxidase reaction product) and endogenous rat IgG (indicated by gold particles). **A, B, C** In the apical domains of this slightly distorted tubular epithelial cell, gp330 is seen in association with clathrin-coated inter-microvillar membrane domains, some of which are extended in areas of loss of tubules. Note that gold particles (denoting rat IgG) co-localize with gp330 indicating that gp330 immune complexes are formed there. Small clusters of gp330-immune complexes are either floating between microvilli or are attached to their surface; gp330-immune complexes likely are shed and dispatched into the lumen. **D, E** Clathrin-coated pits containing gp330-immune complexes are found on the basal side of proximal tubule cell facing the tubular basement membrane; the strict polarized apical expression of gp330 is disturbed. These figures suggest that the immune deposits, which appear in the tubular basement membrane, are caused by a mechanism similar to that operating in the glomerulus. (**A, B, C** and **E** × 48,000; **D** × 75,000.)

Questions and answers

DR. JORDAN J. COHEN (*Dean, School of Medicine, State University of New York, Stony Brook, New York*): You noted that pathogenic antibodies have to traverse the basement membrane to get to the site of antigen expression. Wouldn't normal basement membrane be expected to pose a greater barrier to large antibody molecules?

DR. KERJASCHKI: From Heymann nephritis and several other examples, it appears that there is not an "all or nothing" filtration barrier in the glomerulus. Although the site of the

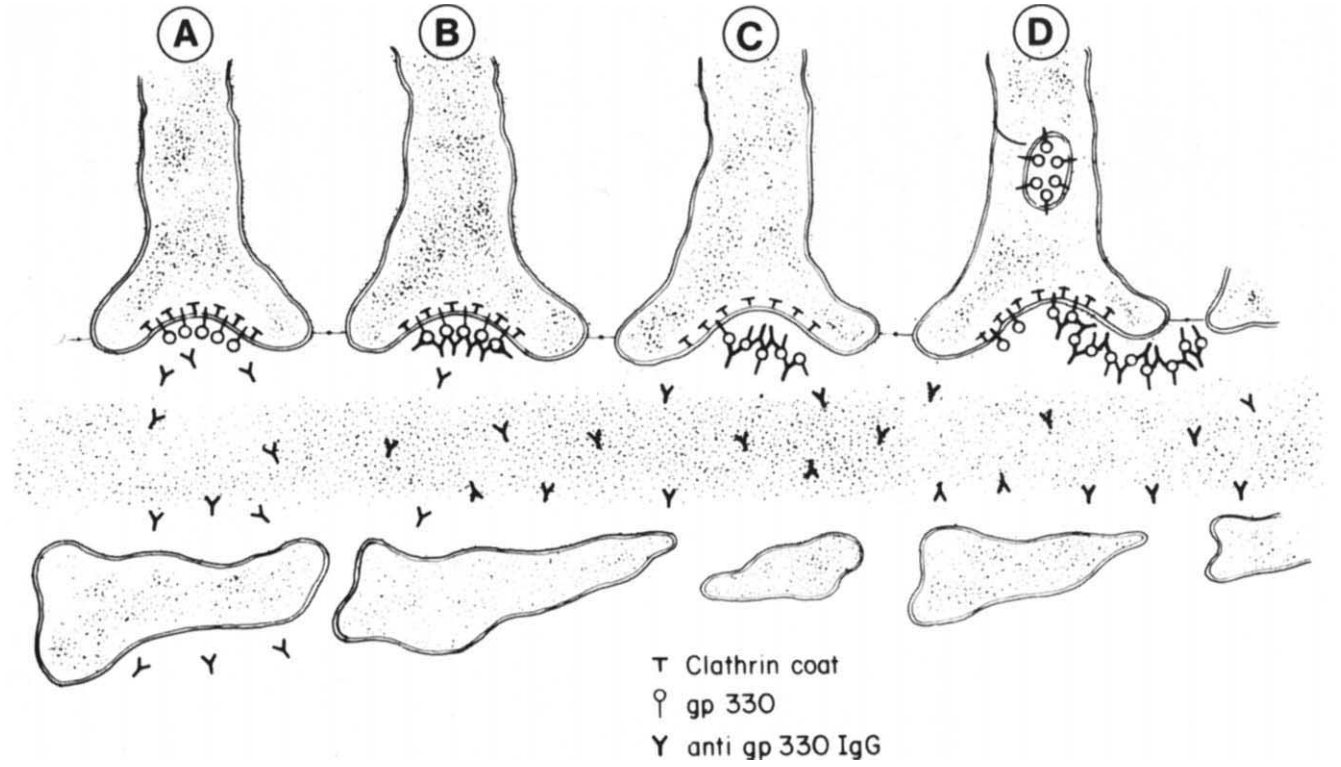

Fig. 11. *Summary of the early events of the formation of an immune deposit in passive Heymann nephritis.* **A** Circulating anti-gp330 IgG (Y) penetrates the GBM and approaches gp330 (♀), a resident membrane glycoprotein of clathrin-coated pits located at the base of the epithelial foot processes. **B** Anti-gp330 IgG binds to its antigen gp330, presumably via a limited number of "pathogenic epitopes" in the coated pits, forming an initial immune complex. **C** The initial immune complex becomes attached to the GBM as early as 15 minutes after injection of the antibody, and is shed but remains in contact with the coated pit. **D** The immune deposit grows in size by repeated cycles of in-situ immune complex formation and shedding into the lamina rara externa until it eventually encroaches on the area of the slit diaphragm. The same result can be obtained if the immune deposit is fixed to one side of the GBM and the foot processes subsequently moved over the immune deposit until positioned under the slit diaphragm. The continued growth of immune deposits appears to require the de-novo synthesis by the podocyte of new molecules of gp330 which, like other membrane proteins, are assumed to be delivered via vesicles that eventually fuse with the cell membrane at the base of the foot processes. (T, clathrin coat; ♀, gp330: Y, anti-gp330 IgG).

barrier in the lamina rara interna has been clearly shown for several molecules, such as dextrans, albumin, and endogenous IgG [78], at least some IgG molecules must penetrate the basement membrane to generate immune deposits on the surface of the glomerular epithelial cells in Heymann nephritis. One could speculate that antibodies or other molecules that interact with components of the basement membrane could penetrate deeper, but no good experimental proof exists to support this speculation. In active Heymann nephritis, Jeraj et al found an antibody system that also interacted with endothelial cells [79]. These researchers speculated that the accessibility of the glomerular epithelial antigens could be enhanced by functional modifications of the endothelial cells [79]. I must emphasize though that these thoughts all are speculations that are not based on firm data at this point.

DR. LEENDERT A. VAN ES (*Professor of Medicine, Department of Nephrology, Leiden University Hospital, Leiden, The Netherlands*): To what extent do eluates from membranous nephropathy in humans follow the behavior of eluates from rats with Heymann nephritis? Do the eluates stain the brush border of proximal tubules, and do they stain coated pits?

DR. KERJASCHKI: The literature contains several reports of experiments in which people have used, for example, eluates from renal cryostat sections from children who had developed de novo membranous nephropathy in a transplanted kidney. These eluates stained the proximal tubular brush border [80]. Molecular analysis, however, has not been conducted.

DR. ANDREW REES (*Professor of Nephrology, Royal Postgraduate Medical School, London, United Kingdom*): You showed that complexes in Heymann nephritis are tightly bound to the GBM, and that gp330 is even more tightly bound than the IgG. Can you tell us anything about the nature of the bonds involved and specifically whether complement is involved?

DR. KERJASCHKI: We have tried to break this interaction by several treatments, such as exposure to detergents, to high- and low-ionic-strength washes, and to urea. None of these manipulations has successfully extracted components of the immune deposits from isolated GBMs. In a very acidic or alkaline milieu, we can extract a major part of the IgG from the immune deposits in isolated GBMs; however, it appears that the gp330 component stays behind. Taken together, these results suggest that we are not dealing with an ionic or hydrophobic interaction. It may well be that in the immune complexes the gp330 molecules somehow covalently bind to currently unknown components of the glomerular basement membrane [59].

The second part of your question is easy to answer. No,

complement is not involved at all. When we injected purified gp330 antibodies into experimental animals and looked at renal tissue after 15 minutes, we found sticking of immune complexes to the GBM but not a trace of complement.

DR. NICOLAOS E. MADIAS (*Chief, Division of Nephrology, New England Medical Center, Boston, Massachusetts*): In various vesicular systems coated with clathrin, H^+ ATPase pumps appear to play a critical role in the function of the vesicle by acidification of its interior [81]. You mentioned that in the proximal tubule, gp330 co-localizes exclusively in the clathrin-coated intermicrovillar domain of the brush-border membranes. My understanding is that it has been a matter of dispute whether H^+ ATPase pumps reside in this particular clathrin-coated domain. Could you please address this point?

DR. KERJASCHKI: We don't know whether there are hydrogen pumps in the proximal tubular brush border that are of relevance to the gp330 molecule. When it comes to the gp330 molecule itself, there appear to be no homologies to any of the known ionic pumps. My educated guess is that gp330 will turn out to be a receptor molecule.

DR. MICHEL MIHATSCH (*Chairman, Department of Pathology, University of Basel, Basel, Switzerland*): Heymann nephritis is one model of membranous nephritis, but other models exist, for example, nephritis induced by injection of cationic compounds. Is gp330 involved in the pathogenesis of this latter model? Are there other endogenous proteins involved in rats or other species? Are there different types of gp330 in different rat species, and is gp330 cationic? If this is so, then there would be only one pathogenetic mechanism.

DR. KERJASCHKI: We have to separate the models for epimembranous glomerulonephritis into two groups: In the first group, endogenous antigens are present in the glomerulus of normal animals as antigenic targets. Currently we only have two examples: the gp330 system and the DPP IV system in the mouse. There is a possibility, as Dr. Verroust mentioned in a previous Nephrology Forum, of getting immune deposits from DPP IV antibodies in the rat [11]. They are, however, very transient and disappear within a few days, unless you create a situation in which the animal has time to develop an immune response against the anti-DPP IV IgG that has been injected. In this situation, the DPP IV immune complexes behave like implanted antigens.

In the second group, experimental lesions are caused only by injection of both heavily cationic compounds and a second round of antibodies directed against them. We have tried to localize gp330—for example, in immune deposits induced by injection of cationic ferritin followed by anti-ferritin antibodies—to answer the question whether the cationic immune complexes are associated with gp330. The answer is that we did not find co-localization. Thus, the mechanism by which cationic compounds stick primarily to the GBM and are subsequently cross-linked by antibodies might be profoundly different from that of gp330, in which an endogenous glomerular antigen is the target of the autoimmunity. To answer your third question, we do not know whether different gp330 species exist (as a result of different gene splicing mechanisms) in different rat strains.

DR. MICHEL GOLDMAN (*Head, Department of Immunology, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium*): Could you elaborate on the genetic susceptibility to membranous nephropathy? More precisely, do you know whether the genetic background in the rat influences the antibody response to gp330 in terms of recognition of pathogenic epitopes?

DR. KERJASCHKI: A very interesting system in which immune deposits are formed was described by Thoenes et al [82]. They transplanted kidneys of Lewis rats into Brown-Norway rats. These two strains were identical in their major histocompatibility antigen and were different only in the minor histocompatibility group. The implanted kidneys from the Lewis rats developed immune deposits, but the kidneys in the Brown-Norway rat recipients did not show any immune deposits. In preliminary experiments in cooperation with Dr. Thoene's group in Munich, we have found that the IgG eluted from the diseased kidney of the donor is specific for gp330. It could be possible that we are looking at two genetically different versions of gp330 in the two rat strains with different epitope structures.

DR. GOLDMAN: Do you know whether the gp330 antigen is involved in membranous nephropathy induced experimentally by chemicals such as mercuric chloride or D-penicillamine?

DR. KERJASCHKI: No.

DR. PIERRE VERROUST (*Directeur de Recherche, INSERM, Hôpital Tenon, Paris, France*): Could you comment on the mechanisms involved in the induction of proteinuria beyond the formation of gp330-anti-gp330 immune complexes?

DR. KERJASCHKI: It is clear that significant proteinuria does not develop in Heymann nephritis without the activation of complement. As I have indicated, the gp330 antibodies by themselves appear to be very inefficient at activating the complement system, or are not able to do so at all, so one of the things we are very interested in is finding out whether a second ancillary complement-activating system exists and defining the antigen(s) involved.

DR. JOHN DONOHOE (*Consultant Nephrologist, Department of Nephrology, Beaumont Hospital, Dublin, Ireland*): What implications do your elegant localization studies have for the natural history of membranous nephropathy? Do they indicate how relatively crude interventions such as the administration of steroids and cyclophosphamide occasionally can be beneficial and on other occasions not so?

DR. KERJASCHKI: The major problem, of course, is that what I have said so far applies only to rats with Heymann nephritis. So far, no one has found a good bridge between the rat and the human. As long as we don't have a catalogue of the molecules serving as antigens in humans, all statements regarding this issue will be speculative.

DR. MADIAS: In rare patients with idiopathic membranous glomerulopathy, anti-GBM disease has developed without pulmonary manifestations. Is there a comparable analogue in rat Heymann nephritis? Can you speculate on the pathogenesis of this evolution?

DR. KERJASCHKI: When Fx1A is used for immunization of rats, the possibility for the development of anti-GBM disease theoretically exists, although I am not aware that such development actually has been detected. Fx1A is a preparation that varies from one batch to the next and from one laboratory to the other. If the glomeruli from the renal cortex aren't sieved out before homogenization, an overlapping syndrome of both anti-GBM disease and Heymann nephritis might be produced.

DR. COHEN: Given the site of origin of the original antigen in

Heymann nephritis, why isn't there more florid tubular manifestation of the disease?

DR. KERJASCHKI: I'm not sure. There is a tubular manifestation that appears early in the course of the disease, but as I mentioned, it is transient.

DR. BRUNO BAGGIO (*Associate Professor of Nephrology, Division of Nephrology, Institute of Internal Medicine, Padova, Italy*): What are the effects of immune complex formation on glomerular glycosaminoglycan metabolism and on the glomerular electrical charge density?

DR. KERJASCHKI: In the case of Heymann nephritis, several people have studied the distribution of anionic charges using different cationic probes that can be seen in the electron microscope. The immune deposits appear to be surrounded by a halo of glycosaminoglycans. The basement membrane outside the immune deposits appears undisturbed [83].

DR. JUDIT NAGY (*Associate Professor of Medicine, Sec. Department of Medicine, University Medical School, Pecs, Hungary*): Is the kidney the only organ in the rat that has the gp330 antigen? When you inject antibody against gp330, do you see binding and formation of immune deposits in any other organ?

DR. KERJASCHKI: No, gp330 is expressed in several types of epithelial cells in coated pits facing their luminal side. Examples are epididymis, yolk sac, and type-II pneumocytes. Extracts of these organs also have been used for immunization, and glomerular immune deposits were obtained. However, extracts from several other organs that do not contain gp330 were found inefficient.

The reason why gp330 antibodies are trapped only in the renal glomerulus after injection may be that this is the only place in the organism where the antibody has direct access from the circulation to the antigen gp330. The gp330 is only separated by fenestrated endothelium and the basement membrane from the capillary lumina.

DR. VAN ES: You mentioned that in the course of Heymann nephritis, gp330 is expressed on the basoluminal side. Theoretically, the possibility exists that the proximal tubular epithelial cell could function as an antigen-presenting cell. Did you look for the expression of class-II MHC molecules during the induction of your model?

DR. KERJASCHKI: No, we have not looked at the expression of MHC molecules.

DR. REES: Your experiments using the recombinant gp330 fusion proteins are very elegant, but I find them a little surprising because most antibodies recognize three-dimensional structures rather than linear sequences of amino acids. Are there precedents for other systems of autoantibodies to linear sequences of amino acids?

DR. KERJASCHKI: There are examples of even shorter stretches than the one we used, such as the N-terminal nonapeptide of the myelin basic protein, which is a major autoimmunologic target in allergic encephalitis [60]. In our case we assume, since we have purified the C14 fusion protein by preparative SDS-electrophoresis and electroelution, that the molecule is badly denaturated and may not refold, but we might be wrong. We don't know how strong the molecular force is that drives the molecule into refolding. As a rule of thumb, it is believed that any peptide that is longer than 12 to 15 amino acids will no longer have a random coil structure but will start to fold.

DR. VERROUST: Could you comment on the nature of the various phases of development of Heymann nephritis?

DR. KERJASCHKI: I believe that after the initial epitope has been hit by the specific antibody, a mechanism is set into motion by which more and more epitopes on the gp330 molecule are exposed and are thus accessible for antibodies. This exposure might stimulate the growth of the immune deposits dramatically. Evidence for a mechanism like this comes from previous experiments in which rats with advanced active Heymann nephritis, which was no longer active and contained only traces of IgG in the immune deposits, were injected with a monoclonal anti-gp330 IgG, which by itself would not induce immune deposits. Within a few minutes, the monoclonal IgG was bound to the "empty" immune deposits. This finding suggested that the corresponding epitope was left behind in the old immune deposit, while the IgG was basically cleared out (unpublished observations). This construction could account for the rapidity in which relapses of the disease occur in humans, but this conclusion is, of course, speculative.

DR. ALEX M. DAVISON (*Consultant Renal Physician, Department of Renal Medicine, St. James's University Hospital, Leeds, United Kingdom*): Human membranous nephropathy clearly is a complex disease and depends on many factors; for instance, an antigen seldom produces a uniform response. Could you speculate on how an understanding of the gp330 system could help in the development of a more rational therapeutic approach to membranous nephropathy?

DR. KERJASCHKI: Speculations would be somewhat utopic at this point, but perhaps at least the Heymann nephritis could be therapeutically manipulated. Assuming you know all the "pathogenic epitopes" of gp330, one approach would be to directly interfere with the binding of circulating antibodies, for example by producing antigenic decoys. Conceptually there are also other possibilities, for example, interference with the adhesion of the immune complexes to the GBM, which might prevent their clearing or—at a distal level—interfering with the complement-mediated glomerular damage.

DR. CHRISTOPHER G. WINEARLS (*Consultant Nephrologist, Renal Unit, Churchill Hospital, Oxford, United Kingdom*): Do the sera of rats with polyclonal stimulation of the immune system include antibodies to gp330?

DR. KERJASCHKI: I don't think so, but certain rabbit strains, when injected with complete adjuvant, started to produce anti-gp330 antibodies that were able to induce passive Heymann nephritis [84]. The rabbits remain healthy, presumably because they do not express gp330 in the glomeruli.

DR. GIUSEPPE REMUZZI (*Head, Laboratory of Kidney Disease, Mario Negri Institute, Bergamo, Italy*): Because of the relative frequency of "de novo" membranous nephropathy in human transplants, I wonder whether gp330 is associated with the MHC complex? Could you comment on the occurrence of "de novo" membranous nephropathy in transplant patients receiving cyclosporine?

DR. KERJASCHKI: First, we do not know any precise relation between MHC molecules and gp330 at this point. In response to your second question, we have no clue from Heymann nephritis.

Reprint requests to Dr. D. Kerjaschki, Institute of Clinical Pathology, University of Vienna, Allgemeines Krankenhaus, Währinger Gürtel 18–20, A-1090 Vienna, Austria

This Forum is in memory of Professor Dr. Wolfgang Thoenes, Institute of Pathology, University of Mainz.

Acknowledgments

Part of the work presented in this Forum was done in collaboration with Dr. Marilyn Farquhar, Division of Cellular and Molecular Medicine, University of California at San Diego. The author is indebted also to his colleagues and friends who have contributed to this work over the years: Dr. A. Miettinen, S. Metone and M. L. Bronson, Drs. G. Dekan, R. Horvat, S. Binder, R. Ullrich, P. Ojha, K. Matsui, A. Howorka, H. Poczewski, B. Langer, and to Dr. J. T. Neale for helpful discussions and for critically reading this manuscript.

This work was supported by a grant from the Fonds zur Förderung der Wissenschaftlichen Forschung (Project P 7742 Med).

Addendum

It recently was discovered that the C14-fragment of gp330 shows extensive amino acid sequence homologies to a heparin-binding membrane protein found in teratocarcinoma cells [85] as well as to an approximately 40 kD extracellular protein that is associated with the α_2-macroglobulin receptor (which by amino acid sequencing was shown to be identical to the LDL-receptor-like protein) [86]. Herz et al suggested that the C14-domain of gp330 is actually a separate molecule of about 40 kD molecular weight, associated with gp330, but which by itself belongs to the LDL-receptor family of membrane proteins. By contrast, several observations indicate that the C14-domain could be an indigenous part of the gp330 molecule. These observations include the finding that in Northern blots of glomerular RNA, the C14-probe selectively binds to an ~10 kb mRNA (which is compatible with a 330 kD protein), while the same probe binds on cultured mesenchymal cells to a 2.5 kb mRNA (compatible with a 40 kD protein) (unpublished observation). Obviously, this conflicting issue will be settled when the entire amino acid sequence of gp330 has been elucidated.

References

1. Ehrenreich T, Churg J: Pathology of membranous nephropathy. *Pathol Annu* 3:145–186, 1968
2. Ponticelli C: Prognosis and treatment of membranous nephropathy. *Kidney Int* 29:927–940, 1986
3. Beregi E, Varga I: Analysis of 260 cases of membranous glomerulonephritis in renal biopsy material. *Clin Nephrol* 2:213–228, 1974
4. Zollinger HU, Mihatsch JM: *Renal Pathology in Biopsy*. Berlin-Heidelberg-New York, Springer-Verlag, 1978, pp 261–278
5. Rosen S: Membranous glomerulonephritis: Current status. *Hum Pathol* 2:209–241, 1971
6. Couser WG: Mechanisms of glomerular injury in immune-complex disease. *Kidney Int* 28:569–583, 1985
7. Cameron JS: Membranous nephropathy: The treatment dilemma. *Am J Kidney Dis* 1:371–375, 1982
8. Heymann W, Hackel DB, Harwood S, Wilson SGF, Hunter JLP: Production of nephrotic syndrome in rats by Freunds adjuvants and rat kidney suspensions. *Proc Soc Exp Biol Med* 100:660–664, 1959
9. Barabas AZ, Lannigan R: Induction of an autologous immune-complex glomerulonephritis in the rat by intravenous injection of heterologous anti-rat kidney antibody. I. Production of chronic progressive immune-complex glomerulonephritis. *Br J Exp Pathol* 55:47–55, 1974
10. Sugisaki TJ, Klassen J, Andres GA, Milgrom FJ, McCluskey RT: Passive transfer of Heymann's nephritis with serum. *Kidney Int* 3:66–73, 1973
11. Verroust P: Nephrology Forum: Kinetics of immune deposits in membranous nephropathy. *Kidney Int* 3:1418–1428, 1989
12. Germuth FG, Rodriguez E: Immunopathology of the renal glomerulus, in *Immune Complex Deposition and Anti-Basement Membrane Disease*. Boston, Little, Brown, 1973, pp 64–73
13. Dixon FJ, Feldman JD, Vazquez JJ: Experimental glomerulonephritis. The pathogenesis of a laboratory model resembling the spectrum of human glomerulonephritis. *J Exp Med* 113:899–937, 1961
14. Edgington TS, Glassock RJ, Dixon FJ: Autologous immune complex pathogenesis of experimental allergic glomerulonephritis. *Science* 155:1432–1434, 1967
15. Edgington TS, Glassock RJ, Dixon FJ: Autologous immune complex nephritis induced with renal tubular antigen: I. Identification and isolation of the pathogenic antigen. *J Exp Med* 127:555–572, 1968
16. Couser WG, Steinmuller DR, Stilmant MM, Salant DJ, Lowenstein LM: Experimental glomerulonephritis in the isolated perfused rat kidney. *J Clin Invest* 62:1275–1287, 1978
17. Van Damme BJC, Fleuren GJ, Bakker WW, Vernier RL, Hoedemaeker PhJ: Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex nephritis. *Lab Invest* 38:502–510, 1978
18. Grupe WE, Kaplan MH: Demonstration of an antibody to proximal tubular antigen in the pathogenesis of experimental autoimmune nephrosis in rats. *J Lab Clin Med* 74:400–409, 1969
19. Ronco P, Melcion C, Geniteau E, Ronco L, Reininger P, Galceran P, Verroust P: Production and characterization of monoclonal antibodies against rat brush border antigens of the proximal convoluted tubule. *Immunology* 53:78–95, 1984
20. Chatelet F, Brianti E, Ronco P, Roland J, Verroust P: Ultrastructural localization by monoclonal antibodies of brush border antigens expressed by glomeruli: II. Extrarenal distribution. *Am J Pathol* 122:512–519, 1986
21. Miettinen A, Törnroth T, Tikkanen I, Virtanen I, Linder E: Heymann nephritis induced by kidney brush border glycoproteins. *Lab Invest* 43:547–555, 1980
22. Kerjaschki D, Farquhar MG: The pathogenic antigen of Heymann nephritis is a glycoprotein of the renal proximal tubule brush border. *Proc Natl Acad Sci USA* 79:5557–5561, 1982
23. Ronco P, Neale TJ, Wilson CB, Galceran M, Verroust P: An immunopathological study of a 330 kD protein defined by monoclonal antibodies and reactive with anti-RTEα5 antibodies and kidney eluates from active Heymann nephritis. *J Immunol* 136:125–130, 1986
24. Kerjaschki D, Farquhar MG: Immunocytochemical localization of the Heymann nephritis antigen (gp330) in glomerular epithelial cells of normal Lewis rats. *J Exp Med* 157:667–686, 1983
25. Bhan AK, Schneeberger EE, Baird LG, Collins AB, Kamata K, Bradford D, Erikson ME, McCluskey RT: Studies with monoclonal antibodies against brush border antigens in Heymann nephritis. *Lab Invest* 53:421–432, 1985
26. Abrass CK: Evaluation of sequential glomerular eluates from rats with Heymann nephritis. *J Immunol* 137:530–537, 1986
27. Bagchus WM, Vos JTWM, Hoedemaeker PhJ, Bakker WW: The specificity of nephritogenic antibodies. III. Binding of anti-Fx1A antibodies in glomeruli is dependent on dual specificity. *Clin Exp Immunol* 63:639–645, 1986
28. Kamata K, Baird LG, Erikson ME, Collins AB, McCluskey RT: Characterization of antigen and antibody specificities involved in Heymann nephritis. *J Immunol* 135:2400–2412, 1986
29. Natori Y, Hyakawa I, Shibata S: Passive Heymann nephritis with acute and severe proteinuria induced by heterologous antibody against renal tubular brush border glycoprotein gp108. *Lab Invest* 55:63–70, 1986
30. Hogendoorn PCW, Bruijn JA, van der Broek LJCM, deHeer E, Foidart JM, Hoedemaeker PhJ, Fleuren GJ: Antibodies to

purified renal tubular epithelial antigens contain activity against laminin, fibronectin and type IV collagen. *Lab Invest* 58:278–286, 1988
31. Assmann KJM, Ronco P, Tangelder MM, Lange WPH, Verroust P, Koene RAP: Comparison of the antigenic targets involved in antibody-mediated glomerulonephritis in the mouse and in the rat. *Am J Pathol* 121:112–119, 1985
32. Ronco P, Van Leer EHG, Chatelet F, Tauc M, Verroust P: Brush border hydrolases expressed by glomerular epithelial cells are target antigens for the formation of immune deposits (*abstract*). *Kidney Int* 31:329a, 1987
33. Natori Y, Hayakawa I, Shibata S: Identification of gp108, a pathogenic antigen of passive Heymann nephritis, as dipeptidyl peptidase IV. *Clin Exp Immunol* 70:434–439, 1987
34. Natori Y, Hayakawa I, Shibata S: Heymann nephritis in rats induced by human renal tubular antigens: Characterization of antigen and antibody specificities. *Clin Exp Immunol*, in press
35. Natori Y, Hayakawa I, Shibata S: Role of dipeptidyl peptidase IV (gp108) in passive Heymann nephritis. *Am J Pathol* 134:405–410, 1989
36. Miettinen A, Törnroth T, Vartio T: Expression of three brush border membrane polypeptides of high molecular weight in rat kidney and other epithelia (*abstract*). *J Cell Biol* 99:106a, 1984
37. Goodyear PR, Mills M, Kaplan BS: Analysis of the Heymann nephritogenic glycoprotein in rat, mouse and human kidney. *Biochem Cell Biol* 64:441–449, 1985
38. Chatelet F, Brianti E, Ronco P, Roland J, Verroust P: Ultrastructural localization by monoclonal antibodies of brush border antigens expressed by glomeruli. I. Renal distribution. *Am J Pathol* 122:500–511, 1986
39. Behar M, Katz A, Silverman M: Biochemical characterization of brush border membrane antigens implicated in the pathogenesis of Heymann nephritis. *Kidney Int* 30:421–430, 1986
40. Behar M, Katz A, Silverman M: A rat cell hybridoma model for study of autologous immune complex nephritis (*abstract*). *Kidney Int* 31:165a, 1987
41. Makker SP, Singh AK: Characterization of the antigen (gp600) of Heymann nephritis. *Lab Invest* 50:287–293, 1984
42. Abrass CK, Border WA, Glassock RJ: Circulating immune complexes in rat with autologous immune complex nephritis. *Lab Invest* 42:1–7, 1980
43. Naruse T, Fukasawa T, Umegae S, Oite S, Miyakawa Y: Experimental membranous glomerulonephritis in rats: Correlation of ultrastructural changes with the serum level of autologous antibody against tubular antigen. *Lab Invest* 39:120–134, 1978
44. Fleuren GJ, Grond J, Hoedemaeker PhJ: The pathogenic role of free-circulating antibody in autologous immune complex glomerulonephritis. *Clin Exp Immunol* 41:205–217, 1980
45. Couser WG: What are circulating immune complexes doing in glomerulonephritis? *N Engl J Med* 300:1230–1232, 1981
46. Hori M, Abrass C: Isolation and characterization of circulating immune complexes from rats with experimental membranous nephropathy. *J Immunol* 144:3849–3855, 1990
47. Ozawa M, Yonezawa S, Sato E, Muramatsu T: A new glycoprotein antigen common to teratocarcinoma, visceral endoderm and renal tubular brush border. *Dev Biol* 91:351–359, 1982
48. Kerjaschki D. Farquhar MG: Pathogenic antigen of Heymann nephritis (gp330): Identification, isolation, and localization, in *Nephrology* (vol 1), edited by Robinson RR, New York, Springer-Verlag, 1985, pp 560–574
49. Kanalas JK, Makker SP: Identification of the rat Heymann nephritis autoantigen (gp330) as a receptor site for plasminogen (*abstract*). *Fed Proc* 5:908A, 1991
50. Mendrick DL, Chung D, Rennke H: Heymann antigen gp330 demonstrates affinity for fibronectin, laminin, and type I collagen and mediates rat proximal tubule epithelial cell adherence to such matrices in vitro. *Exp Cell Res* 188:23–35, 1990
51. Kerjaschki D, Ojha PP, Susani M, Horvat R, Binder S, Hovorka A; Hillemanns P, Pytela R: A β_1-integrin receptor for fibronectin in human kidney glomeruli. *Am J Pathol* 134:481–489, 1989
52. Pietromonaco S, Kerjaschki D, Binder S, Ullrich R, Farquhar MG: Molecular cloning of a cDNA encoding a major pathogenic domain of the Heymann nephritis antigen gp330. *Proc Natl Acad Sci (USA)* 87:1811–1815, 1990
53. Raychowdhury R, Niles J, McCluskey RT, Smith JA: Autoimmune target in Heymann nephritis is a glycoprotein with homology to the LDL receptor. *Science* 244:1163–1165, 1989
54. Kerjaschki D, Noronha-Blob L, Sacktor B, Farquhar MG: Microdomains of distinctive glycoprotein composition in the kidney proximal tubule brush borders. *J Cell Biol* 98:1505–1513, 1984
55. Rodman JSD, Kerjaschki D, Merisko EC, Farquhar MG: Presence of an extensive clathrin coat on the apical plasmalemma of the rat kidney proximal tubule cell. *J Cell Biol* 98:1630–1636, 1984
56. Leung CCK: Isolation, partial characterization and localization of a rat renal tubular glycoprotein. Antibody-induced birth defects. *J Exp Med* 156:372–384, 1982
57. Sahali D, Mulliez N, Chatelet F, Dupuis R, Ronco P, Verroust P: Characterization of a 280 kD protein restricted to the coated pits of the renal brush border and the epithelial cells of the yolk sac: Teratogenic effect of the specific monoclonal antibodies. *J Exp Med* 167:213–218, 1988
58. Allegri L, Brianti E, Chatelet F, Manara GC, Ronco P, Verroust P: Polyvalent antigen-antibody interactions are required for the formation of electron dense immune deposits in passive Heymann nephritis. *Am J Pathol* 126:1–6, 1986
59. Kerjaschki D, Miettinnen A, Farquhar MG: Initial events in the formation of immune deposits in passive Heymann nephritis. *J Exp Med* 166:109–128, 1987
60. Clayton JP, Gammon GM, Ando DG, Kono DH, Hood L, Sercarz EE: Peptide specific prevention of experimental allergic encephalomyelitis. Neonatal tolerance induced to the dominant T cell determinant of myelin basic protein. *J Exp Med* 169:1681–1691, 1990
61. Marx J: Testing of autoimmunity therapy begins. *Science* 254:27–28, 1991
62. Neale TJ, Wilson CB: Glomerular antigens in Heymann nephritis: Reactivity of eluted and circulating antibody. *J Immunol* 128:323–330, 1982
63. Goldstein JL, Anderson RGW, Brown MS: Coated pits, coated vesicles, and receptor mediated endocytosis. *Nature* 279:679–683, 1979
64. Doxsey SD, Kerjaschki D, Farquhar MG: A large membrane glycoprotein (gp330) is a resident of coated pits of several absorptive epithelia (*abstract*). *J Cell Biol* 97:178a, 1983
65. Buc MH, Condamine H, Kerjaschki D: Rat Heymann nephritis antigen is closely related to brushin, a glycoprotein present in early mouse embryo epithelia. *Ann Inst Pasteur* 138:707–722, 1987
66. Camussi G, Brentjens JR, Noble B, Kerjaschki D, Malavasi F, Roholt OA, Farquhar MG, Andres GA: Antibody-induced redistribution of Heymann antigen on the surface of cultured glomerular visceral epithelial cells: Possible role in the pathogenesis of Heymann glomerulonephritis. *J Immunol* 135:2409–2416, 1985
67. Camussi G, Kerjaschki D, Gonda M, Nevins T, Rielle JC, Brentjens J, Andres GA: Expression and modulation of surface antigens in cultured rat glomerular visceral epithelial cells. *J Histochem Cytochem* 37:1675–1687, 1989
68. Camussi G, Salvidio G, Biesecker G, Brentjens J, Andres GA: Heymann antibodies induce complement-dependent injury of rat glomerular visceral epithelial cells. *J Immunol* 139:2906–2918, 1987
69. Andres G, Brentjens JR, Caldwell PRB, Camussi G, Matsuo S: Formation of immune deposits and disease. *Lab Invest* 55:510–520, 1987
70. Camussi G, Noble B, van Liew JB, Brentjens J, Andres GA: Pathogenesis of passive Heymann nephritis: Chlorpromazine inhibits antibody-mediated redistribution of cell surface antigens and prevents development of the disease. *J Immunol* 163:2127–2134, 1986
71. Biesecker G, Noble B, Andres GA, Koffler D: Immunopathogenesis of Heymann's nephritis. *Clin Immunol Immunopathol* 33:333–346, 1984
72. Kerjaschki D, Horvat R, Binder S, Susani M, Dekan G, Ojha PP, Hillemanns P, Ulrich W, Donini U: Identification of a

400-kD protein in the brush borders of human kidney tubules that is similar to gp330, the nephritogenic antigen of rat Heymann nephritis. *Am J Pathol* 129:183–191, 1987

73. NARUSE T, KITAMURA D, MIYAKAWA Y, SHIBATA S: Deposition of renal tubular epithelial antigens along the renal glomerular capillary walls of patients with membranous glomerulonephritis. *J Immunol* 110:1163–1169, 1973
74. DOUGLAS MFS, RABIDEAU DP, SCHWARTZ MM, LEWIS EJ: Evidence on autologous immune complex nephritis. *N Engl J Med* 305:1326–1329, 1981
75. ZANETTI M, MANDET C, DUBOUST A, BEDROSIIAN J, BARIETY J: Demonstration of a passive Heymann-nephritis-like mechanism in human kidney transplants. *Clin Nephrol* 15:272–288, 1981
76. NILES J, COLLINS B, BAIRD L, ERIKSON M, BRADFORD D, PAN G, HSIUNG CK, SCHNEEBERGER EE, BHAN A, MCCLUSKEY RT: Antibodies reactive with a renal glycoprotein and deposits in membranous nephritis (*abstract*). *Kidney Int* 31:338A, 1987
77. FUKATSU A, YUZAWA Y, OLSON L, MILLER J, MILGROM M, ZAMLAUSKI-TUCKER MJ, VAN LIEW J, CAMPAGNARI A, NIESEN N, PATEL J, DOI T, STRIKER G, STRIKER L, MILGROM F, BRENTJENS J, ANDRES GA: Interaction of antibodies with human glomerular epithelial cells. *Lab Invest* 61:389–403, 1989
78. FARQUHAR MG: The primary glomerular filtration barrier—basement membrane or epithelial slits? *Kidney Int* 8:197–291, 1975
79. JERAJ K, VERNIER RL, SISSON SP, MICHAEL AF: A new glomerular antigen in passive Heymann's nephritis. *Br J Exp Pathol* 43:65–485, 1984
80. ANTIGNAC C, HINGLAIS N, GUBLER MC, GAGNADOUX MF, BROYER AM, HABIB R: De novo membranous glomerulonephritis in renal allografts in children. *Clin Nephrol* 30:1–16, 1988
81. STONE D: Proton-translocating ATPases: Issues in structure and function. *Kidney Int* 33:767–774, 1988
82. THOENES GH, PIELSTICKER K, SCHUBERT G: Transplantation induced immune complex disease in rats with unilateral manifestation in the allografted kidney. *Lab Invest* 41:321–333, 1979
83. HOGENDOORN PCW, DE HEER E, WEENING JJ, DAHA MK, HOEDEMAEKER PHJ, FLEUREN GJ: Glomerular capillary wall change and antibody binding in passive HN. *J Lab Clin Med* 111:150–157, 1988
84. MIETTINEN A, TÖRNROTH T, LINDER E: Immunologically mediated tubulo-interstitial nephritis in rabbits (*abstract*). *Scan J Immunol* 4:759, 1975
85. FURUKAWA T, OZAWA M, HUANG RP, MURAMATSU T: A heparin binding protein whose expression increases during differentiation of embryonal carcinoma cells to parietal endoderm cells: Cloning and sequencing analysis. *J Biochem* 108:297–302, 1990
86. HERZ J, GOLDSTEIN JL, STRICKLAND OK, HO YK, BROWN MS: 39-kDa protein modulates binding of ligands to low density lipoprotein-related protein/α_2-macroglobulin receptor. *J Biol Chem*, in press